PURIFICATION AND PROPERTIES OF CEPHALOSPORINASE FROM *PSEUDOMONAS AERUGINOSA*

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Cephalosporin β-lactamase (cephalosporinase; CSase) was purified from a strain of *Pseudomonas aeruginosa* resistant to β-lactam antibiotics. The purified enzyme preparation gave a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and its molecular weight was about 34,000. The specific activity was 49.7 μmoles/minute/mg of protein of the purified enzyme for the hydrolysis of cephaloridine. The optimal pH and optimal temperature were about 8.0 and 40°C, respectively. Its isoelectric point was 8.7. The enzyme activity was inhibited by iodine, some divalent ions, and some semisynthetic β-lactam antibiotics, including cephamycin derivatives such as moxalactam and YM09330. Mouse antiserum obtained against the purified enzyme showed no cross-reaction with other types of β-lactamase in neutralization test.

β-Lactamases have been considered to have a significant role in the high resistance of organisms to penicillins and cephalosporins.[1,2,3] Many workers have investigated the enzymological properties of β-lactamases produced by Gram-positive and Gram-negative bacteria, including *Pseudomonas aeruginosa*.[4~7]

P. aeruginosa strains have been isolated with increasing frequency from clinical specimens.[8,9,10] On the other hand, new β-lactam antibiotics have been developed that possesses greater potency against Gram-positive and Gram-negative bacteria including *P. aeruginosa*.[11,12,13]

Among a large number of resistant strains from clinical sources, we selected 5 strains of *P. aeruginosa* (GN918, GN10362, GN10367, GN10368, GN10370) capable of producing cephalosporinase in a large amount. This paper deals with the enzymological, physicochemical and immunological properties of purified enzyme from *P. aeruginosa* GN10362. Furthermore, we examined the inhibitory activity of a number of β-lactam antibiotics, including newly developed ones, against the purified enzyme and compared the properties of the β-lactamases from other Gram-negative bacteria.

Methods

Bacterial Strains

P. aeruginosa GN918, GN10362, GN10367, GN10368 and GN10370 were isolated from clinical sources, and they were the stock cultures in this laboratory.

Media

Brain heart infusion broth (Difco, USA) and MÜELLER-HINTON agar (Nissui, Tokyo) were used. Medium B was used for large-scale culture and consisted of 2 g of yeast extract, 10 g of peptone, 8 g of $Na_2HPO_4 \cdot 12H_2O$, 2 g of KH_2PO_4, 1.2 g of $(NH_4)_2SO_4$, 2 g of glucose, 0.4 g of $MgSO_4$, and 1,000 ml of distilled water. Peptone water consisted of 10 g of peptone, 5 g of NaCl, and 1,000 ml of distilled water.

Determination of the Minimum Inhibitory Concentration

The minimum inhibitory concentration was determined by an agar dilution technique. Overnight cultures of test strains in peptone water were diluted to a final concentration of 10^6 cells/ml, and 5 μl of each culture was inoculated by an inoculator (Microplanter; Sakuma, Tokyo) on the MÜELLER-HINTON agar plates containing serial two-fold dilutions of a drug. The minimum inhibitory concentration (MIC) was determined after overnight incubation at 37°C.

Drugs

Cephaloridine (CER), cefazolin (CEZ), cephalothin (CET), cephalexin (CEX), penicillin G(PCG), ampicillin (APC), carbenicillin (CPC), cloxacillin (CLX), and methicillin (MTC) were commercially available materials. Other compounds were received as gifts from manufactures: cefotiam (CTM), cefamandole (CMD), cefoperazone (CFP), cefuroxime (CXM), cefotaxime (CTX), ceftizoxime (CZX), SCE-1365, cefoxitin (CFX), cefmetazole (CMZ), moxalactam (MXA), YM09330, cefsulodin (CFS), clavulanic acid (CVA), CP-45899 and MK-0787.

Culture and Harvesting of the Organism

The organism was grown overnight at 37°C in 3 liters of brain heart infusion broth. The culture was diluted 3-fold by medium B and grown at the same temperature under aeration for 3 hours. At the moment of incubation, ampicillin was added to final concentration of 300 μg/ml as an inducer for the production of CSase, and the incubation was continued. After 3 hours of incubation the cells were harvested by centrifugation and washed once with 0.05 M sodium phosphate buffer (pH 7.0). Then the cell suspension in 100 ml of 0.05 M sodium phosphate buffer (pH 7.0) was disrupted with a UR-150P supersonic vibrator (Tomy Seiko, Tokyo) at 75 W in an ice water bath. The broken cells were centrifuged at 13,000$\times g$ for 30 minutes at 4°C, and the resulting supernatant was used for the enzyme assay.

Preparation of Column

A CM-Sephadex C-50 (Pharmacia, Sweden) column and a Sephadex G-100 (Pharmacia, Sweden) column were prepared as described previously.[14]

Enzyme Assay

β-Lactamase activity was determined by either a spectrophotometric method, measuring the decrease in absorbance at an appropriate wave-length of the substrate (100 μM) in a temperature-controlled spectrophotometer (Beckmen model 24) at 30°C as described by HIRAI *et al.*[15] or a modification of the NOVICK microiodometric method,[16] using penicillin as a substrate.

One unit of enzyme activity was defined as the amount of enzyme that hydrolyzed 1 μmole of a substrate in 1 minute at 30°C, in 0.05 M sodium phosphate buffer (pH 7.0).

Protein Determination

The concentration of protein in column eluate was estimated by measuring the absorbance at 280 nm. A more accurate estimation was carried out by LOWRY's method[17] with bovine serum albumin as the standard.

Determination of Molecular Weight

The molecular weight of the purified enzyme was determined by sodium dodecyl sulfate-discontinuous gel electrophoresis according to WEBER and OSBORN,[18] using albumin (molecular weight 68,000), ovalbumin (45,000), chymotripsinogen A (25,000), and cytochrome c (12,500) as the molecular weight standards. The gel concentration was 10%.

Determination of Isoelectric Point

The isoelectric point of the enzyme was determined by electrofocusing. The purified sample was used for electrofocusing column chromatography employing Ampholine electrofocusing equipment, model LKB 8100 (LKB Produkter AB, Stockholm-Bromma, Sweden). The carrier ampholyte used gave a pH gradient spectrum between pH 3.5 and pH 10.0. Electrofocusing was carried out below 4°C in an electrolysis column with 110 ml capacity. The total amount of carrier ampholytes was 2.5 ml. After focusing for 45 hours, the contents of the column were cut into 3 ml fractions, and each fraction was assayed for its β-lactamase activity and pH.

Production of Antiserum

Mouse antiserum was produced against the purified preparation from GN10362. Male ICR mice were used for the production of antiserum. The purified enzyme preparation (50 μg of protein) from GN10362 was dissolved in 0.1 ml of saline and emulsified with the same volume of complete Freund's adjuvant. The enzyme solution (0.2 ml) was intraperitoneally injected into a mouse. After 2 and 4 weeks following the first injection, booster injections containing 50 μg of protein were administered intraperitoneally. Antiserum was collected 2 weeks after the last injection.

Immunological Method

The effect of the antiserum on the activity of β-lactamases was examined by neutralization test of the hydrolyzing activity of the enzyme. The enzyme solution (0.1 ml, 0.94 units/ml) was incubated with various amount of antiserum in 0.1 ml 0.05 M phosphate buffer (pH 7.0) at 37°C for 1 hour and then left at 4°C for 18 hours. The mixture solution was centrifuged at 3,000 rpm for 10 minutes and the residual β-lactamase activity of the supernatant was assayed spectrophotometrically, using cephaloridine (100 μM) as a substrate.

Results

Substrate Profile of the Cephalosporinase from *P. aeruginosa*

We selected several strains of *P. aeruginosa* that were highly resistant to β-lactam antibiotics. From these resistant strains, we chose five *P. aeruginosa* strains (GN918, GN10362, GN10367, GN10368, GN10370), whose β-lactam resistance were not mediated by plasmids. The crude enzymes from these five strains showed almost the same substrate profile (Table 1), and *P. aeruginosa* GN10362 was used for further studies.

Table 1. Substrate profile of CSase produced by *P. aeruginosa*.

Substrate	Relative rate of hydrolysis (%)[a]				
	GN918	GN10362	GN10367	GN10368	GN10370
CER	100	100	100	100	100
CEZ	132	150	134	132	85
CET	495	449	597	534	355
CEX	32	37	25	37	50
CTM	32	30	27	25	10
CFP	4	4	5	8	5
CXM	0	0	0	0	0
CFS	0	0	0	0	0
CMD	0	0	0	0	0

[a] The rate of hydrolysis is expressed in percentage of the hydrolysis of CER: the concentration of substrate was 100 μM.

Table 2. Summary of the purification of CSase from *P. aeruginosa* GN10362.

Stage No.	Procedure	Total activity[a] (units)	Specific activity (units/mg of protein)	Purification factor	Total recovery (%)
1	Ultrasonic disintegration	1083.6	0.46	1.00	100
2	SM fraction	921.2	0.47	1.02	85.0
3	CM-Sephadex C-50	384.0	24.2	52.60	35.4
4	Sephadex G-100	319.3	49.7	108.00	29.5

[a] The enzyme activity was assayed spectrophotometrically with cephaloridine as a substrate.

Purification Procedure

All manipulations were carried out at 4°C, and the pH of all buffers used was 7.2. The specific CSase activity of the purified enzyme was 49.7 units/mg of protein (Table 2).

Physicochemical Properties of the Purified Enzyme

The purified enzyme preparation gave a single protein band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the molecular weight of the purified enzyme was estimated to be 34,000. The isoelectric point of the enzyme determined by electrofocusing was 8.7.

Resistance Levels of GN10362 to Various β-Lactam Antibiotics

The resistance levels of *P. aeruginosa* GN10362 to a number of β-lactam antibiotics are shown in Table 3. The strain was found to be highly resistant to almost drugs used.

Table 3. Kinetics of hydrolysis of various β-lactam antibiotics by CSase from *P. aeruginosa* GN10362 and its resistance levels.

Substrate	*Km*(μM)	*Ki*[a](μM)	V_{max}[b]	MIC (μg/ml)[c]
CER	167	—[d]	100	>800
CEZ	667	—	222	>800
CET	71	—	139	>800
CEX	42	—	64	>800
CTM	83	—	12	>800
CMD	15	—	3	>800
CFP	23	—	5	3.1
CXM	—	0.003	<1	>800
CTX	—	0.27	<1	6.3
CZX	—	2.0	<1	12.5
SCE-1365	—	0.24	<1	6.3
CFX	—	0.23	<1	>800
CMZ	—	0.10	<1	>800
MXA	—	0.39	<1	6.3
YM09330	—	0.15	<1	400
CFS	—	100	<1	0.8
PCG	34	—	29	>800
APC	—	10	<1	>800
CPC	—	1.5	<1	25
CLX	—	0.006	<1	>800
MTC	—	0.002	<1	>800
CVA	—	>100	<1	200
CP-45899	—	>100	<1	>800
MK-0787	—	3.13	<1	1.56

[a] Values of *Ki* were determined by using cephalothin as a substrate.
[b] Rates of hydrolysis are expressed in percentage of hydrolysis of cephaloridine.
[c] See text for details; MIC, minimum inhibitory concentration.
[d] —; None.

Table 4. Effects of inhibitors and divalent ions on the activity of CSase from *P. aeruginosa* GN10362.

Inhibitors and ions	Concentration (mM)	Inhibition (%)
Iodine	0.05	100
	0.1	100
*p*CMB[a]	0.1	2.7
	0.5	12.4
	1.0	20.0
Hg^{2+}	0.5	100
	1.0	100
Zn^{2+}	0.5	47
	1.0	60
Co^{2+}	0.5	27
	1.0	53
Cu^{2+}	0.2	31

[a] *p*CMB; *p*-chloromercuribenzoate.

Table 5. Effects of various β-lactam antibiotics on the activity of CSase from *P. aeruginosa* GN10362.

Antibiotics	I_{50} (μM)[a]	Antibiotics	I_{50} (μM)[a]
CXM	7	APC	340
CTX	30	CPC	1.4
CZX	500	CLX	1.1
SCE-1365	41	MTC	2
CFX	40	MK-0787	340
CMZ	32	CP-45899	8
MXA	<1	CVA	>1000
YM09330	<1		

[a] I_{50}; The concentration of the antibiotics which inhibits 50% of the enzyme activity.

Enzymatic Properties of the Purified Enzyme

The temperature of 40°C was optimal for the enzyme activity. The optimal pH was about 8.0 and the enzyme showed less than 50% of its maximum activity at pH 6.0 (data is not shown) when cephalothin was used as a substrate.

The MICHAELIS constant (*Km*), maximum rate of hydrolysis (relative V_{max}) and the dissociation constant of the enzyme-inhibitor complex (*Ki*) are shown in Table 3. *Km* Values and relative V_{max} values were obtained with cephaloridine, cefazolin, cephalexin and penicillin G. Cefotiam, cefoperazone and cefamandole were less rapidly hydrolyzed by the enzyme than the above antibiotics. On the other hand, cefotaxime, ceftizoxime, SCE-1365 and penicillins with exception of penicillin G showed low V_{max} values, and a high affinity for the enzyme (low *Km* value). Clavulanic acid, CP-45899 and MK-0787 also showed resistance to the hydrolysis by the enzyme. However, the former two compounds had no affinity to the enzyme. This enzyme had neither affinity nor hydrolyzing activity to cefsulodin.

Inhibitory Action of Inhibitors, Divalent Ions and Several Antibiotics

A number of enzyme inhibitors, divalent ions and several antibiotics, which are resistant to the hydrolysis of the CSase, were tested for their inhibitory effect on the activity of the purified enzyme. The enzyme was preincubated in distilled water with each of the inhibitors, divalent ions and antibiotics at indicated concentrations for 10 minutes at 30°C, and then the remaining enzyme activity was assayed with 100 μM cephalothin as a substrate (Tables 4 and 5). Iodine and Hg^{2+} ion completely inhibited the enzyme activity but *p*-chloromercuribenzoate failed to inhibit the enzyme activity at the concentration of 1 mM. Among the antibiotics used, moxalactam and YM09330 showed the strongest inhibitory action on the enzyme activity (low I_{50} value) and the inhibition percents of each drug were 94 and 87, respectively, at the concentration of 1 μM.

Immunological Property of the Purified Enzyme

The neutralizing capacity of the antiserum against the purified enzyme was checked on various types of β-lactamase. As shown in Table 6, the antiserum completely neutralized the enzyme activity of the CSase from *P. aeruginosa* not only GN10362 but also GN918 and GN10367. However other types of β-lactamase, including 4 types of penicillinase, were not affected by the antiserum of *P. aeruginosa* GN10362.

Table 6. Cross reaction of the antiserum of CSase from *P. aeruginosa* GN10362.

Enzyme source	Inhibition %[a]	Enzyme source	Inhibition %[a]
P. aeruginosa GN10362	100	*P. morganii* GN5407	0
P. aeruginosa GN918	100	*P. vulgaris* GN7919	0
P. aeruginosa GN10367	100	*B. fragilis* GN11478	0
C. freundii GN7391	0	Rms212 (PCase type I)	0
E. cloacae GN7471	0	Rms213 (PCase type II)	0
S. marcescens GN10857	0	Rte16 (PCase type III)	0
P. rettgeri GN4430	0	Rms139 (PCase type IV)	0

[a] See text for details.

Discussion

The β-lactamase from *P. aeruginosa* GN10362 was purified about 108-fold with CM-Sephadex C-50 and Sephadex G-100 column chromatography, and was found to be homogeneous and pure by the polyacrylamide gel electrophoretic analysis. The isoelectric point of the purified enzyme is 8.7, this value being almost the same as those of *P. aeruginosa* strains reported by YAGINUMA *et al.*[7] (pI=

8.7), and LABIA *et al.*[5] (pI=8.66).

As indicated by *Km* and V_{max} values, this enzyme has a strong affinity and hydrolyzing activity on cephalosporin derivatives and a poor activity for penicillin derivatives and cefuroxime-type cephalosporins. Thus the β-lactamase from *P. aeruginosa* GN10362 presents a CSase profile as for typical CSase described by SAWAI *et al.*,[19] but this enzyme is considered to be a different type from those of *P. cepacia*,[15] *P. vulgaris*[20] and *B. fragilis*,[21] which we classified as cefuroximase. On the other hand, the neutralization tests of the antiserum against the CSase from GN10362 showed that the enzyme was highly specific to *P. aeruginosa* species, because anti-GN10362 serum could only cross-react with CSase from *P. aeruginosa* GN10362, GN918 and GN10367, but did not with other β-lactamases.

The inhibitory action of several newly developed β-lactam antibiotics and penicillins against the activity of the CSase was examined. Moxalactam and YM09330 showed the strongest inhibitory effect on the enzyme activity. The enzyme activity was almost completely inhibited at the concentration of 1 μM. Although clavulanic acid had no inhibitory effect on the enzyme, CP-45899 inhibited the enzyme almost to the same degree as cefuroxime, *Ki* values of both compounds being similar (>100 μM). Further investigation should be needed to clarify such a difference.

P. aeruginosa GN10362 capable of producing CSase was resistant to various β-lactam antibiotics. However the stability or affinity of such drugs to the CSase of *P. aeruginosa* GN10362 was not correlated with their antibacterial activities to the CSase-producing strains. Cefsulodin, which has no affinity or inhibitory activity to the enzyme, showed the highest antibacterial activity. On the other hand, YM09330 and cefuroxime, possesing high inhibitory action to the enzyme, had no antibacterial activity against the strain. From these observations it seems likely that other factors, such as penetrability of the compound, are involved in resistance mechanisms.

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